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A Ubiquitin-Proteasome Pathway Represses the *Drosophila* Immune Deficiency Signaling Cascade

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Summary

Background: The inducible production of antimicrobial peptides is a major immune response in *Drosophila*. The genes encoding these peptides are activated by NF-κB transcription factors that are controlled by two independent signaling cascades: the Toll pathway that regulates the NF-κB homologs, Dorsal and DIF; and the IMD pathway that regulates the compound NF-κB-like protein, Relish. Although numerous components of each pathway that are required to induce antimicrobial gene expression have been identified, less is known about the mechanisms that either repress antimicrobial genes in the absence of infection or that downregulate these genes after infection.

Results: In a screen for factors that negatively regulate the IMD pathway, we isolated two partial loss-of-function mutations in the *SkpA* gene that constitutively induce the antibacterial peptide gene, *Diptericin*, a target of the IMD pathway. These mutations do not affect the systemic expression of the antifungal peptide gene, *Drosomycin*, a target of the Toll pathway. *SkpA* encodes a homolog of the yeast and human Skp1 proteins. Skp1 proteins function as subunits of SCF-E3 ubiquitin ligases that target substrates to the 26S proteasome, and mutations affecting either the *Drosophila* SCF components, Slimb and dCullin1, or the proteasome also induce *Diptericin* expression. In cultured cells, inhibition of SkpA and Slimb via RNAi increases levels of both the full-length Relish protein and the processed Rel-homology domain.

Conclusions: In contrast to other NF-κB activation pathways, the *Drosophila* IMD pathway is repressed by the ubiquitin-proteasome system. A possible target of this proteolytic activity is the Relish transcription factor, suggesting a mechanism for NF-κB downregulation in *Drosophila*.

Introduction

One defense that the fruit fly, *Drosophila melanogaster*, employs against microbial infection is the inducible expression of antimicrobial peptides [1]. Flies produce at least seven types of antimicrobial peptides that are active against different classes of microbes. These peptides are synthesized in both surface epithelial tissues and the fat body, an analog of the mammalian liver. Peptides produced in the fat body are secreted into the insect hemolymph to high concentrations. Studies on how the antimicrobial peptide genes are induced by infection have provided insight into insect defense strategies and have demonstrated surprising parallels with the control of innate immune responses in mammals [1].

The induction of antimicrobial peptide genes in response to Gram-negative bacterial infection is mediated by Relish, a *Drosophila* NF-κB-like protein that, like the mammalian compound NF-κB proteins, p100 and p105, is composed of a DNA binding Rel-homology domain and an inhibitory ankyrin-repeat domain [2]. Relish activity is controlled by a signaling pathway initially characterized by a mutation in the *immune deficiency* (*imd*) gene that blocks the induction of antimicrobial peptide genes after Gram-negative bacterial infection and that renders flies highly susceptible to Gram-negative bacterial infection [3]. Genetic screens for mutations with similar phenotypes identified factors that function with IMD in a pathway that shares similarities with the Tumor Necrosis Factor Receptor-1 (TNFR-1) pathway in mammals: IMD is similar to the mammalian Receptor Interacting Protein (RIP) kinase that interacts with TNFR-1 [4]; downstream of IMD, *Drosophila* Transforming Growth Factor-β Activating Kinase 1 (dTAK1) [5] regulates a *D. melanogaster* IκB kinase (DmIKK) complex, characterized by the structural component DmIKKγ/Kenny [6] and the kinase DmIKKβ/Immune Response Deficient 5 (Ird5) [7] that appears to phosphorylate and trigger Relish cleavage [8]. This cleavage generates a free Rel-homology domain that translocates into the nucleus and a stable ankyrin-repeat domain that remains in the cytoplasm [9, 10]. Although p100 and p105 processing is mediated by the ubiquitin-proteasome pathway (reviewed in [11]), Relish cleavage does not require proteasome activity, but it is dependent on the Dredd caspase [9, 12]: the presence of a potential caspase cleavage site in the Relish linker domain suggests that Relish is processed by a caspase protease [13]. Recent RNA interference (RNAi)-based studies also indicate that the *Drosophila* Fas-Associated Death Domain-containing protein (dFADD) links IMD to Dredd [14]. The first recognition molecule linked to the IMD pathway is PGRP-LC, a membrane-bound protein that is required to activate the IMD pathway after infection [15–17].

To identify factors that repress the IMD pathway, we screened for mutations that constitutively induce expression of the antibacterial peptide gene, *Diptericin*, which is tightly regulated by the IMD pathway (reviewed in [18]). We isolated two independent loss-of-function mutations in the *SkpA* gene that upregulate *Diptericin* but that do not affect *Drosomycin*, a target of the Toll pathway. *SkpA* encodes a homolog of the mammalian and yeast Skp1 proteins that are components of Skp1/Cullin/F-box protein (SCF)-E3 ubiquitin ligases (re-

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viewed in [19]), and we used genetic studies to determine that an SCF complex and the proteasome block *Diptericin* expression in uninfected flies. Inhibiting this SCF complex in cultured *Drosophila* cells increases the steady-state levels of both the full-length and the processed Relish Rel-homology domains. Consequently, we hypothesize that the ubiquitin-proteasome system negatively regulates the IMD pathway by degrading Relish and that this is a mechanism for downregulating Relish activity.

Results

The *J6* and *G49* Mutations Induce *Diptericin* Expression

In wild-type flies, the gene encoding the antibacterial peptide *Diptericin* is tightly controlled by the IMD pathway (reviewed in [18]). Therefore, to identify genes that normally function to repress the IMD pathway, we screened 2,000 *yellow*, *white* (*y*,*w*) F1 male progeny from male flies mutagenized with ethyl methanesulfonate for constitutive expression of a *Green Fluorescent Protein* (*GFP*) reporter gene under the control of the *Diptericin* promoter [20]. Two males, *J6* and *G49*, expressed *Diptericin-GFP*, and this gene was constitutively expressed in larvae and adults in homozygous lines derived from these males (Figure 1A). Although flies carrying the *J6* and *G49* mutations are viable and fertile at 25°C, *G49* is pupal lethal at 29°C, indicating temperature-sensitive phenotypes associated with this mutation (see below). To determine if the *J6* and *G49* mutations affect the actual *Diptericin* gene, we used RNA gel blots to monitor *Diptericin* expression in mutant larvae and flies. Quantification of the RNA gel blots show that *J6* larvae constitutively express *Diptericin* to 10% of the levels induced in *y,w* larvae 8 hr after infection by pricking with the Gram-negative bacterial species *Erwinia carotovora carotovora* 15 (*Ecc*15) (Figure 1B). *Diptericin* expression in *G49* larvae varies from 30% to 45% of the levels in infected *y,w* larvae, with larvae raised at 29°C showing higher levels of expression. In G49 adults maintained at 29°C, *Diptericin* transcripts accumulate to 75% of the levels induced by *Ecc*15 infection of *y,w* adults. In contrast to *Diptericin*, *Drosomycin* levels in mutant larvae and adults are equivalent to or only slightly higher than the levels in uninfected individuals, indicating that the *J6* and *G49* mutations specifically activate *Diptericin* (Figures 1B). We also determined that flies heterozygous for either of the two mutations and the parental *y,w* chromosome do not express *Diptericin* constitutively, indicating that the two mutations are recessive (Figure 1C and data not shown). Flies heterozygous for both the *G49* and *J6* mutations, however, do express *Diptericin* constitutively, demonstrating that the two mutations belong to a single complementation group (Figure 1C).

The *G49* Mutation Prolongs IMD Pathway Activation after Infection

Drosophila maintains independent pathways for mediating immune responses to different types of pathogens: Gram-negative bacterial infections induce *Diptericin* via the IMD pathway, and fungal infections induce *Drosomycin* expression via the Toll pathway (reviewed in [18]). To determine if the constitutive *Diptericin* mutations affect activation of either of these pathways by infection, we infected *G49* adults maintained at 29°C by either pricking them with *Ecc*15 or by exposing them to spores from the fungus *Beauvaria bassiana*. We then quantified *Diptericin* and *Drosomycin* expression in the infected flies over time on RNA gel blots. Based on the average of two independent experiments, *Diptericin* expression peaks 6 hr after *Ecc15* infection in wild-type adults; in *G49* adults, however, *Diptericin* expression mounts significantly until 24 hr after infection, and levels remain high 48 hr after infection (Figure 2A). After *B. bassiana* infection, *Drosomycin* is induced with similar kinetics in wild-type and *G49* adults, but to much higher levels in the *G49* adults (Figure 2B). Relish activation via the IMD pathway partially induces *Drosomycin* expression [12]; consequently, we suspect that the high *Drosomycin* levels in fungally infected *G49* adults is caused by the simultaneous activation of both the Toll and IMD pathways in these flies. The dominant, gain-of-function mutation of the *Toll* gene, $Toll^{10b}$, constitutively induces *Drosomycin* expression [21], and we also determined that the *G49* mutation does not reduce *Drosomycin* levels in $Toll^{10b}$ larvae (Figure 2C). Based on these *Diptericin* and *Drosomycin* expression patterns, we conclude that the *G49* mutation does not inhibit activation of either the IMD or the Toll pathways by infection but does inhibit downregulation of the IMD pathway after infection.

J6 and *G49* Are Mutations in the *SkpA* Gene

Using recombination mapping, we resolved that the *J6* and *G49* mutations are tightly linked to the *y* locus on the proximal tip of the X chromosome (data not shown). To further localize the two mutations, we used deletions to determine that *J6* falls in the area defined by the overlap of *Df(1)74k24.1*, *Df(1)svr*, and *Df(1)su(s)83*, placing it in cytological region 1B10 near the *Dredd* gene [12, 22, 23] (Figure 3A). We then identified two lethal P-element insertions in the Bloomington stock center collection, *l(1)G0389* and *l(1)G0109*, which were previously mapped near this region, that do not complement the constitutive *Diptericin* expression in the *J6* and *G49* lines (Figure 3B and data not shown). By sequencing DNA flanking the P elements in the two insertion lines, we ascertained that both elements lie within 200 bp of each other in the 5′ untranslated region of the *SkpA* gene (data not shown) (GadFly: Genome Annotation Database of *Drosophila* [http://hedgehog.lbl.gov:8002/cgi-bin/annot/query/]). To confirm that *J6* and *G49* are mutations in *SkpA*, we showed that a wild-type *SkpA* transgene on the second chromosome (T.D. Murphy, personal communication) suppresses the constitutive *Diptericin* expression phenotype in *G49* flies (Figure 3B). Finally, we compared *SkpA* sequences from the original *y,w* line and the *J6* and *G49* lines. The *J6* and *G49* lines each contain a point mutation in the *SkpA* gene that generates a single amino acid change in the SkpA protein: *J6*, renamed $SkpA^{J6}$, converts threonine 98 to an isoleucine, and *G49*, renamed $SkpA^{G49}$, replaces glutamic acid 101 with a lysine (Figure 3C). These alleles are hypomorphic mutations of *SkpA* since the P-element

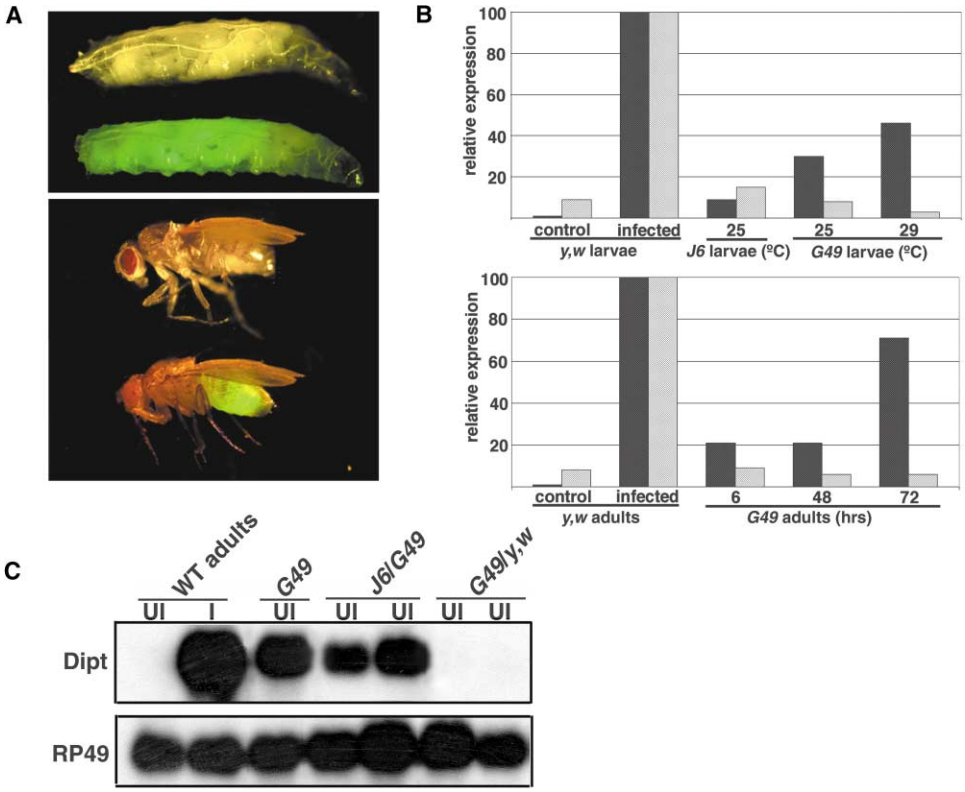


Figure 1. The *J6* and *G49* Mutations Constitutively Induce the *Diptericin* Gene

(A) Two X-linked mutations, *J6* and *G49*, were isolated by screening for the constitutive expression of a *Diptericin-GFP* reporter gene in larvae and adults. The upper panel shows a wild-type larva on the top and a GFP-expressing larva from the *J6* line on the bottom. The lower panel shows a wild-type fly on the top and a GFP-expressing adult from the *G49* strain on the bottom.

(B) Northern blot quantification of *Diptericin* and *Drosomycin* expression shows that, in *J6* and *G49* larvae, *Diptericin* levels, indicated by black bars, range from 10%–45% of the levels induced 8 hr after Gram-negative bacterial infection of larvae from the parental *y,w* strain. In *G49* adults maintained at 29°C, *Diptericin* expression reaches 70% of the levels observed in *y,w* adults, 8 hr after Gram-negative bacterial infection. In contrast to *Diptericin*, *Drosomycin*, indicated by gray bars, is not strongly expressed in *J6* and *G49* larvae and adults. The age of G49 adults is shown in hr.

(C) A Northern blot shows that flies heterozygous for the *J6* and *G49* mutations display the constitutive *Diptericin* phenotype, demonstrating that these two mutations belong to a single complementation group. Flies heterozygous for *G49* and the *y,w* chromosome do not express *Diptericin*, demonstrating that the *G49* mutation is recessive. Similar results were obtained for the *J6* mutation (data not shown). Two independent RNA samples from *J6/G49* and *G49/y,w* flies are shown, and the levels of *Ribosomal protein 49* (*RP49*) transcripts in each sample were assayed for a loading control. UI: uninfected adults; I: adults sampled 8 hr after Gram-negative bacterial infection.

insertions are pupal lethal at 25°C. $SkpA^{G49}$ is pupal lethal at 29°C, and homozygous $SkpA^{G49}$ adults transferred to 29°C express *Diptericin* at similar levels as flies heterozygous for $SkpA^{G49}$ and either the P-element insertions or deletions that remove *SkpA* (Figure 3B and data not shown). At 29°C, therefore, $SkpA^{G49}$ behaves like a null mutation, which probably reflects the significant change from the negatively charged glutamic acid to the positively charged lysine in this allele.

An SCF Complex and the Proteasome Repress *Diptericin* Expression

The *SkpA* gene encodes a protein that is highly similar to Skp1 proteins in humans and yeast (Figure 3D) [24]. Skp1 proteins are components of SCF ubiquitin ligases that target substrates to the proteasome, and crystal structures of human Skp1 complexed with the F-box protein Skp2 and the cullin protin Cul1 have been solved [25, 26]. $SkpA^{J6}$ and $SkpA^{G49}$ both affect a conserved region of SkpA that corresponds to helix 5 of Skp1; helix 5 forms part of the core interface between Skp1, the F-box region of Skp2, and the amino-terminal domain of Cul1, with some amino acids in this helix making direct contact with residues in Skp2 and Cul1 (Figure 3D). This suggests that the $SkpA^{J6}$ and $SkpA^{G49}$ mutations disrupt interactions between SkpA and the F-box protein and cullin components of an SCF complex. Protein interaction studies indicate that SkpA functions with the F-box protein Slimb and the Cullin-like protein dCullin1 (dCul1) in a *Drosophila* SCF complex [24]. In support of this model, we confirmed that $slimb^{1}$ [27] and $dcul1^{l(2)02074}$ mutant larvae, as well as larvae carrying the *DTS5* mutation, a dominant-negative mutation that affects the β6 subunit of the 26S proteasome [28], express *Diptericin* at levels comparable to those in the *SkpA* mutants (Figure 4A). To further test the *DTS5* phenotype, we used the UAS-Gal4 system [29] to overexpress a *UAS-DTS5* transgene [30] in larval fat bodies: DTS5

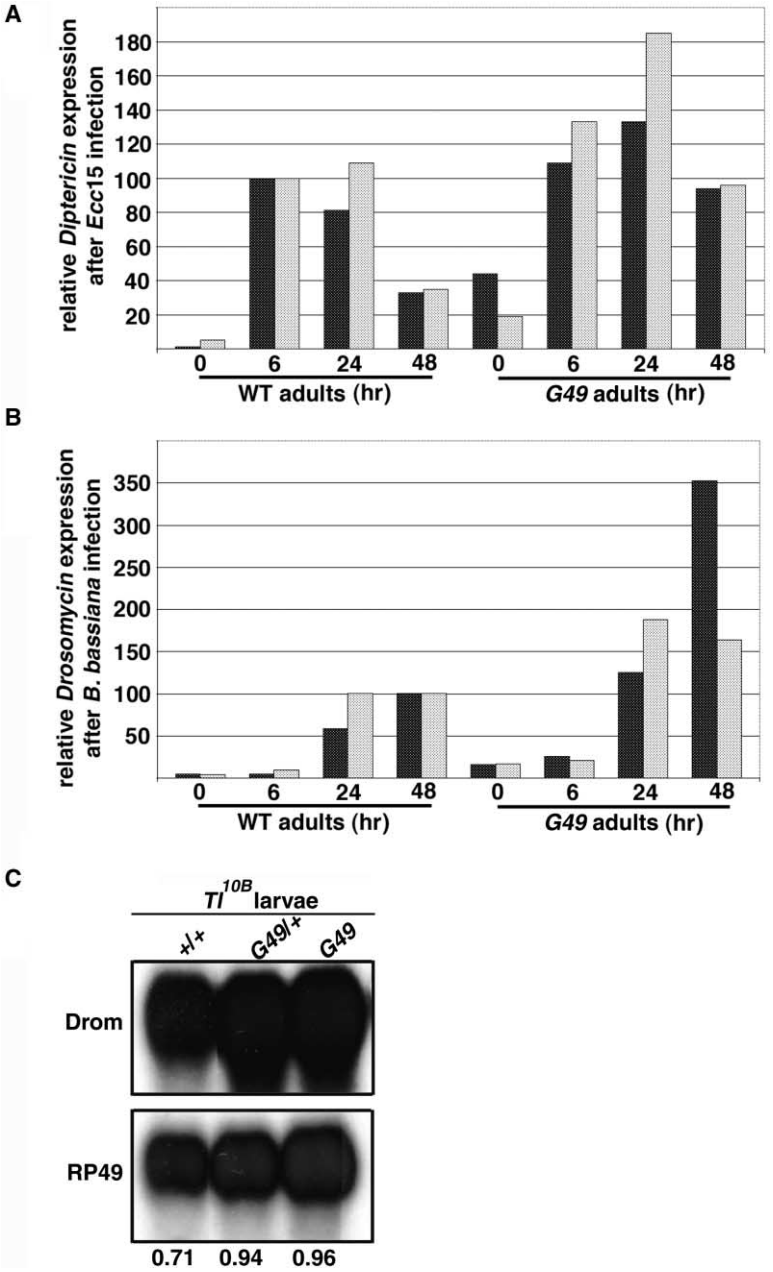


Figure 2. The *G49* Mutation Does Not Affect Activation of Either the IMD or Toll Pathways

(A) Northern blot quantifications of two independent experiments, with one experiment represented by black bars and the second experiment represented by gray bars, demonstrate that infections with the Gram-negative bacterial strain *Ecc*15 induce *Diptericin* expression to high levels in *G49* adults and that this expression persists at higher levels than in wild-type flies. The wild-type and *G49* flies used for these experiments were maintained at 29°C both before and after infection.

(B) Similarly, *B. bassiana* fungal infections induce *Drosomycin* expression to high levels in *G49* adults maintained at 29°C.

(C) The Tl^{10B} mutation is a dominant mutation that activates the Toll receptor and induces constitutive *Drosomycin* expression [21]. A Northern blot shows that the constitutive *Drosomycin* expression in Tl^{10B} larvae that do not carry the *G49* mutation (+/+) is not reduced in either female larvae that are heterozygous for the *G49* mutation (*G49*/+) or male larvae that are hemizygous for the *G49* mutation (*G49*). The ratio of the *Drosomycin* signal to the *RP49* signal in each sample is shown below.

overexpression induces *Diptericin* to levels that are comparable to those generated by bacterial infection with *Ecc15* (Figure 4A). We also generated flies heterozygous for mutations at both the *SkpA* and *slimb* loci: these flies constitutively express *Diptericin*, indicating a synergistic interaction between *SkpA* and *slimb* (Figure 4B). The constitutive *Diptericin* expression in the $slimb^{1}$, $dcul1^{l(2)02074}$, and *DTS5* mutants and the interaction between *SkpA* and *slimb* together suggest that an $SCF^{SkpA/dCul1/Slimb}$ ubiquitin ligase represses *Diptericin* expression by targeting a regulatory factor for degradation by the 26S proteasome.

The IMD Pathway Is Repressed by an SCF Complex

To determine if the constitutive *Diptericin* expression in the SCF complex mutants is mediated through the IMD pathway, we examined *Diptericin* levels in larvae homozygous for mutations in either *SkpA*, or *slimb* and various genes of the IMD pathway: $SkpA^{J6}$;imd^{1} and $SkpA^{G49}$;$dtak1^{1}$ double mutants display constitutive *Diptericin* expression; although, *Diptericin* levels are slightly reduced in the $SkpA^{G49}$;$dtak1^{1}$ larvae (Figure 5A). Mutations in *Dmlkk*γ, *Dmlkk*β, and *Relish*, however, completely block *Diptericin* expression in the $SkpA^{J6}$ background, and a *Dredd* mutation completely blocks *Diptericin* expression in the $slimb^{1}$ background (Figures 5A and 5B). The constitutive *Diptericin* expression observed in *SkpA* and *slimb* mutants, therefore, does not require IMD and dTak1, but it is dependent on the DmIKK complex, Dredd, and Relish. These results imply that, in wild-type flies, the $SCF^{SkpA/dCul1/Slimb}$ negatively regulates the IMD pathway by targeting one of these factors, or

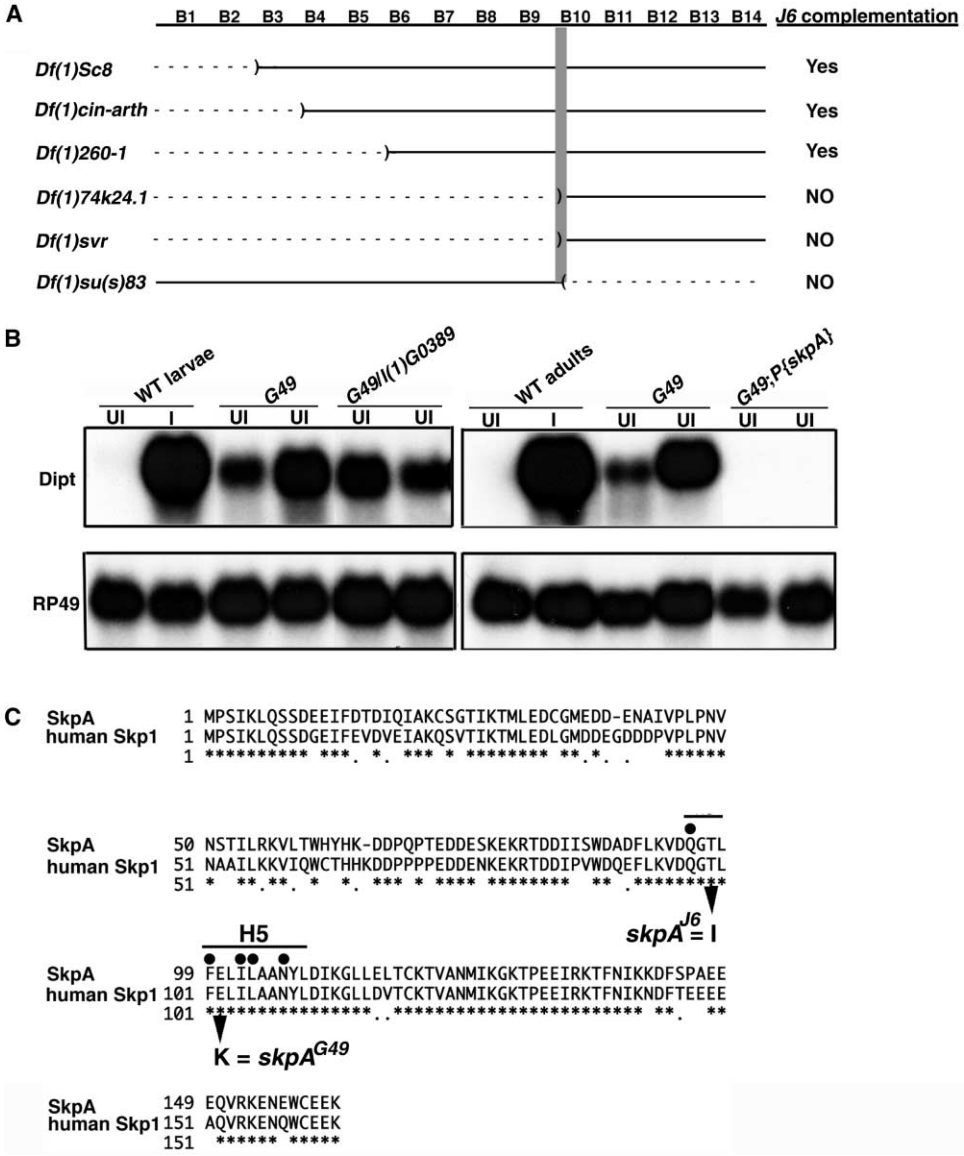


Figure 3. *J6* and *G49* Are Mutations in the *Drosophila SkpA* Gene

(A) Recombination mapping linked the *J6* mutation to the *y* gene on the distal tip of the X chromosome (data not shown). Subsequent deletion mapping determined that the *J6* mutation is in the 1B10 region of the X chromosome, marked by the gray bar, that is uncovered by the deletions *Df(1)74k24.1*, *Df(1)su(s)83*, and *Df(1)svr*. Numbered bands of X chromosome segment 1B are indicated on top.

(B) Two lethal P-element insertions, *l(1)G0389* and *l(1)G0109*, mapped near this region and failed to complement the constitutive *Diptericin-GFP* expression induced by the *J6* mutation (data not shown). Sequencing of the regions flanking the *l(1)G0389* and *l(1)G0109* P-element insertions determined that both insertions were in the 5′ untranslated portion of the *SkpA* gene (data not shown). In the left panel, Northern blot analysis shows that larvae heterozygous for *l(1)G0389* and the *G49* mutation constitutively express *Diptericin*, indicating that *l(l)G0389* also fails to complement the *G49* mutation. The Northern blot in the right panel demonstrates that the constitutive *Diptericin* expression in *G49* adults is suppressed by a wild-type *SkpA* transgene (T.D. Murphy, personal communication), confirming that *G49* is a mutation in *SkpA*. Two independently isolated RNA samples are shown in consecutive lanes for each genotype. UI: uninfected larvae; I: larvae sampled 8 hr after Gram-negative bacterial infection.

(C) Sequence analysis of the *SkpA* gene in the *J6* and *G49* lines shows that each carries an independent point mutation that leads to an amino acid substitution in the SkpA protein. The *J6* mutation, renamed $SkpA^{J6}$, changes threonine (T) 98 to an isoleucine (I). The G49 mutation, renamed $SkpA^{G49}$, replaces glutamic acid (E) 101 with a lysine (K). Amino acids conserved between SkpA and the human Skp1 protein are marked with an asterix; similar amino acids are marked with a small dot. Amino acids corresponding to helix 5 (H5) in the human Skp1 protein are indicated with a line, and amino acids in human Skp1 helix 5 that make direct contact with either the F-box protein, Skp2, or the cullin protein, Cul1, are marked with large circles [25, 26].

an additional unidentified component of the IMD pathway, for degradation by the proteasome. In contrast to fat body cells, the IMD pathway is the primary regulator of all antimicrobial genes, including *Drosomycin*, in surface epithelial tissues [20]. A *Drosomycin-GFP* transgene is constitutively expressed in tracheal cells but not

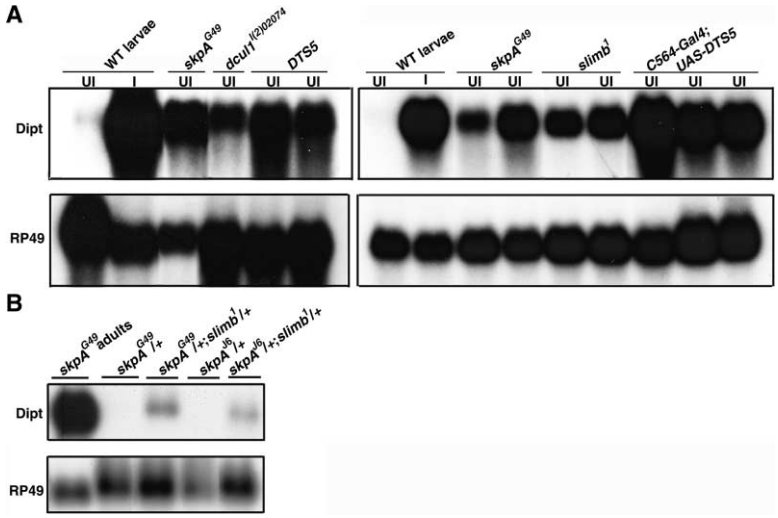


Figure 4. Other SCF Complex and Proteasome Mutations Also Activate *Diptericin* Expression

(A) Northern blot analysis shows that mutations affecting the *dcul1*, *slimb*, and 26S proteasome β5 subunit (*DTS5*) genes, like mutations in *SkpA*, induce constitutive *Diptericin* expression. Overexpression of the dominant *DTS5* mutation using the UAS-Gal4 system with the *c564* driver that is active in larvae [7] generates levels of *Diptericin* comparable to those in larvae 8 hr after Gram-negative bacterial infection. Multiple, independent RNA samples are shown for most genotypes. UI: uninfected larvae; I: larvae sampled 8 hr after Gram-negative bacterial infection.

(B) Synergistic interactions between mutations in *SkpA* and *slimb* are demonstrated by low levels of constitutive *Diptericin* expression in flies heterozygous for either $SkpA^{J6}$ or $SkpA^{G49}$ and the $slimb^{1}$ mutation.

in fat body cells of $slimb^{1}$ mutant larvae; this expression pattern further demonstrates that the IMD pathway, but not the Toll pathway, is constitutively activated when the $SCF^{SkpA/dCul1/Slimb}$ complex is compromised (Figure 5C).

Inhibiting SCF Activity Increases Relish Steady-State Levels

Although our genetic results do not allow us to differentiate between the DmIKK complex, Dredd, Relish, or other unidentified downstream components of the IMD pathway as targets of the ubiquitin-proteasome pathway, the mammalian Relish homolog, P105, is regulated by an SCF complex that contains the Slimb homolog β-TrCP/$E3RS^{IκB}$ (reviewed in [11]). Consequently, we used RNA-mediated interference (RNAi), which we previously demonstrated as an effective technique for specifically inhibiting targeted proteins [10], in cultured *Drosophila* S2 cells to test for interactions between the $SCF^{SkpA/dCul1/Slimb}$ complex and Relish. We first blocked SkpA and Slimb activity in S2 cells via RNAi. We then induced transient expression of a full-length Relish protein, modified by an N-terminal FLAG tag, in the same S2 cells and monitored the effects of the *SkpA* and *slimb* RNAi treatments on FLAG-Relish protein stability using Western blots and anti-FLAG antibodies.

Reducing Slimb activity, in the absence of LPS stimulation, visibly increases steady-state levels of both full-length Relish and the active N-terminal Rel-homology

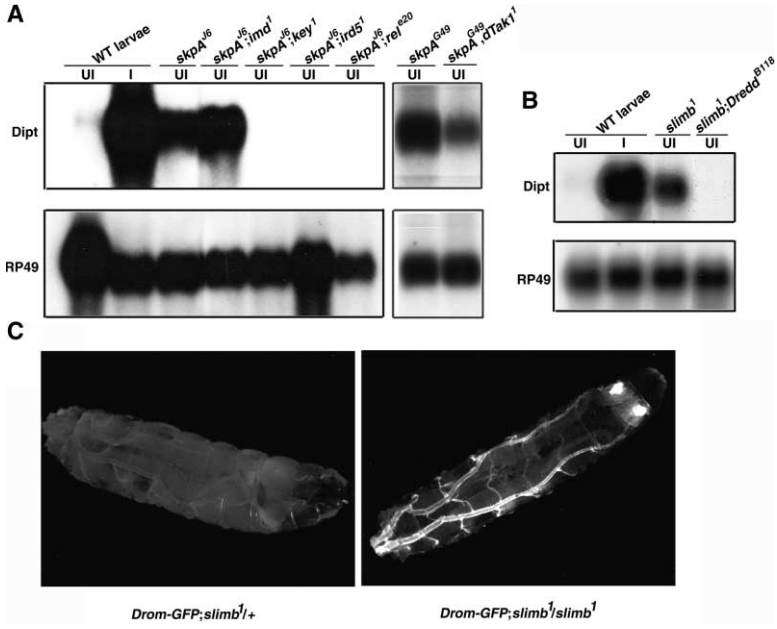
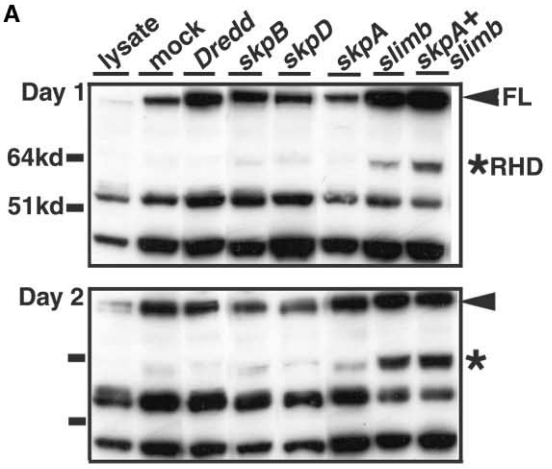


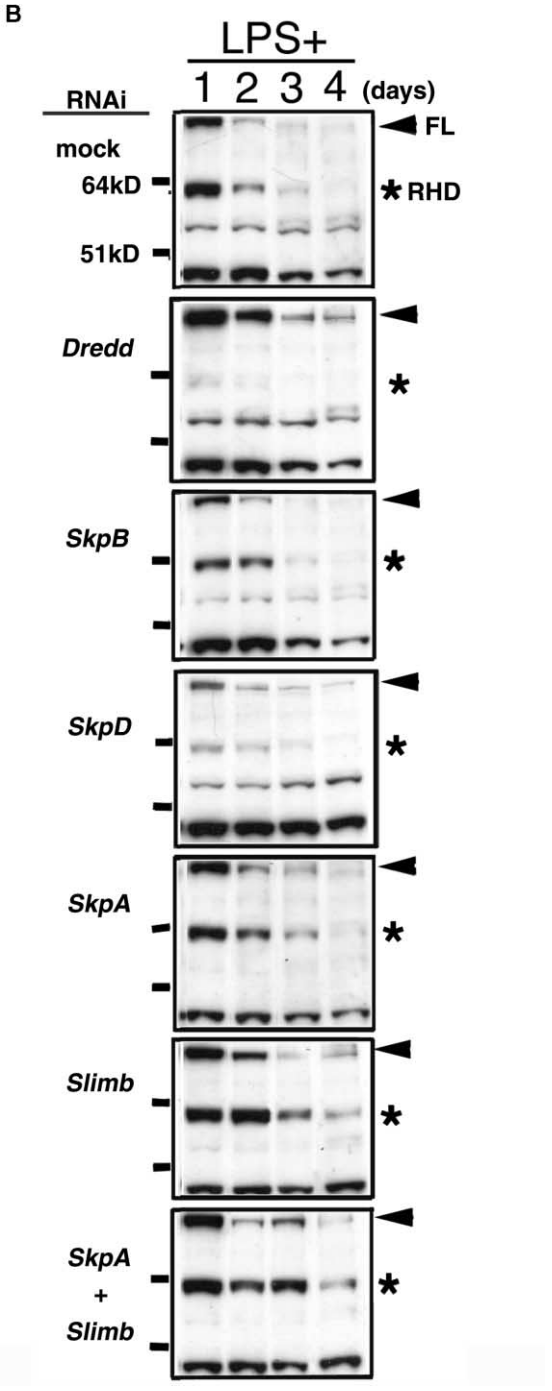
Figure 5. Constitutive Antimicrobial Gene Expression in *SkpA* and *slimb* Mutants Requires Downstream Components of the IMD Pathway

(A) *Diptericin* expression levels in larvae doubly homozygous for either the $SkpA^{J6}$ or the $SkpA^{G49}$ mutation and mutations affecting the IMD pathway demonstrate that the constitutive *Diptericin* expression induced by the *SkpA* mutations is not affected by the imd^{1} and $dTak1^{1}$ mutations but is suppressed by the *ird5* mutation of DmIKKβ, $DmIKK\beta^{ird5}$; the key^{1} mutation of DmIKKγ, $DmIKK\gamma^{key1}$; and the $Relish^{e20}$ mutations.

(B) Similar epistatic analysis shows that the $Dredd^{B118}$ mutation blocks the $slimb^{1}$-induced *Diptericin* expression. UI: uninfected larvae; I: larvae sampled 8 hr after Gram-negative bacterial infection.

(C) Although the *Drosomycin* gene is predominantly regulated by the Toll pathway in the fat body, the IMD pathway regulates *Drosomycin* expression in trachea and other epithelial tissues [20]. A *Drosomycin-GFP* reporter gene is constitutively expressed in the trachea of larvae homozygous for the $slimb^{1}$ mutation, shown on the right, confirming that the $slimb^{1}$ mutation activates the IMD pathway. A larva carrying the *Drosomycin-GFP* (Drom-GFP) reporter gene that is heterozygous for the $slimb^{1}$ mutation is shown on the left.





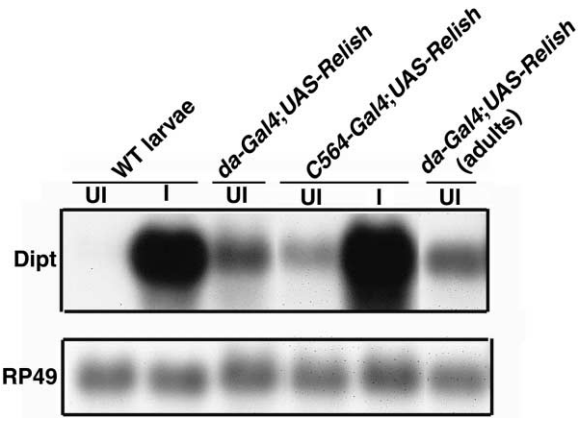


Figure 7. *Relish* Overexpression Induces Constitutive *Diptericin* Expression

The expression of a *UAS-Relish* transgene in both larvae and adults by different Gal4 drivers is sufficient to induce low levels of *Diptericin* expression, indicating that Relish is constitutively activated in the absence of infection. UI: uninfected larvae; I: larvae sampled 8 hr after Gram-negative bacterial infection.

domain; levels of both polypeptides are further increased by inhibiting Slimb and SkpA simultaneously (Figure 6A). This effect is specific since RNAi of the SkpA homologs, SkpB and SkpD, does not increase Relish levels. Dredd RNAi does increase Relish levels at day 1, but this is probably because Dredd inhibition blocks Relish processing (Figure 6A) [9]. Previous studies show that Relish processing in S2 cells is induced by lipopolysaccharide (LPS) and requires Dredd activity [9]. As expected, therefore, RNAi of Dredd blocks LPS-induced Relish processing (Figure 6B). Simultaneous RNAi of SkpA and Slimb in the presence of LPS, however, results in higher steady-state levels of the Rel-homology domain up to 4 days after Relish induction (Figure 6B). Higher levels of the Rel-homology domain after SkpA and Slimb RNAi could be caused by increased processing of full-length Relish. However, because full-length Relish levels also mount, we favor the explanation that Rel-homology domain turnover is reduced. Although the Slimb and SkpA RNAi treatments appear to inhibit Relish turnover, Relish levels do eventually diminish (Figure 6B). This suggests that RNAi efficiency decreases with time, possibly due to degradation of the transfected double-stranded RNA. These RNAi experiments indicate that the constitutive antimicrobial gene expression in *SkpA* and *slimb* mutant flies is

Figure 6. RNAi-Mediated Inhibition of *SkpA* and *slimb* Increases Relish Protein Levels

(A) Western blot analysis of total protein extracts shows that N-terminal FLAG-tagged Relish protein produced by the transient expression of a copper-sensitive *FLAG-Relish* transgene in *Drosophila* S2 cells is present at higher levels following RNAi of *SkpA* and *slimb*. In particular, the Relish Rel-homology domain (RHD) of approximately 62 kd is only detectable after individual *SkpA* and *slimb* or simultaneous *SkpA* and *slimb* RNAi. Nonspecific proteins of around 50 kDa that react with the α-flag antibodies serve as loading controls. "Lysate" indicates an extract from untreated S2 cells; "mock" indicates cells transfected with an empty RNAi vector.

(B) Western blot analysis of total protein extracts from cells exposed to LPS after transient expression of Relish show that RHD levels remain higher in cells after RNAi of *SkpA* and *Slimb*, up to 4 days after transient Relish expression. As expected, RNAi of *Dredd* blocks Relish processing. FL: full-length Relish protein; RHD: Relish Rel-homology domain.

caused by higher Relish levels, and they suggest that the $SCF^{SkpA/dCul1/Slimb}$ complex represses the IMD pathway by promoting the degradation of both full-length and processed Relish proteins.

Overexpressing *Relish* Induces *Diptericin* Expression

If the constitutive antimicrobial gene expression in flies carrying mutations that affect the $SCF^{SkpA/dCul1/Slimb}$ complex or proteasome is due to higher Relish levels, it implies some level of steady-state Relish activation. Low levels of the Rel-homology domain were previously reported in nuclear extracts from unstimulated S2 cells, and these low levels indicate that Relish is constitutively processed [9]. Figure 7 shows that increasing Relish levels in larvae and adults via the Gal4-UAS system is sufficient to induce low levels of *Diptericin* expression. These results indicate that Relish is constitutively processed and activated to some level, supporting our hypothesis that Relish activity, in the absence of infection, is countered by ubiquitination and degradation.

Discussion

Recent studies of antimicrobial peptide gene expression and resistance to infection in *Drosophila* have clarified the Toll and IMD signaling cascades: these two pathways control antimicrobial peptide gene expression via NF-κB transcription factors; they are similar to the pathways that regulate NF-κB in mammals; and they appear to function independently, without any common components (reviewed in [18]). Based on genetic studies, we have determined that an SCF-E3 ubiquitin ligase and the 26S proteasome repress the IMD pathway, possibly by promoting the turnover of constitutively active Relish protein.

In mammals, current studies indicate that SCF complexes containing the Slimb homolog β-TrCP/E3RS$^{I\kappa B}$ activate NF-κB by catalyzing the degradation of inhibitory IκB proteins via the 26S proteasome (reviewed in [11]). In addition, two NF-κB factors, p50 and p52, are generated from precursor proteins, p105 and p100, respectively, that are ubiquitinated and partially degraded by the 26S proteasome, and the $SCF^{\beta TrCp}$ complex is implicated in both p105 processing and complete degradation [31]. In flies, the IκB homolog, Cactus, is degraded and the NF-κB homologs, Dorsal and DIF, are released when the Toll pathway is activated during development and after infection (reviewed in [1]). Cactus contains a sequence similar to the IκB degradation motif that is recognized by the $SCF^{\beta TrCP}$ complex [32], and it is proposed that Slimb mediates Cactus degradation during development [33]. Mutations in *SkpA*, however, do not inhibit the Toll pathway, suggesting that another *Drosophila* Skp1 homolog might function with Slimb to regulate Cactus degradation.

Although Cactus may be targeted for degradation by an SCF complex, to date, there was no evidence that a ubiquitin ligase or the proteasome function in the IMD pathway: Relish, the NF-κB homolog in the IMD pathway, is a compound Rel-protein, similar to mammalian p100 and p105, but is probably processed by a caspase [9, 12, 13]. Nevertheless, mutations affecting the $SCF^{SkpA/Slimb/dCul1}$ complex and the 26S proteasome induce constitutive antimicrobial gene expression, and this gene expression is dependent on Relish and components of the IMD pathway, the DmIKK complex and Dredd, that are probably directly involved in Relish processing. Consequently, we predict that the ubiquitin-proteasome pathway represses Relish activity by degrading one of these factors. Overexpression of *DmIKK*β and *Dredd* leads to low levels of *Diptericin* expression [5], indicating that they could be targets of the $SCF^{SkpA/Slimb/dCul1}$ complex. Based on our observation, however, that inhibiting SkpA and Slimb activity in cultured *Drosophila* cells increases levels of full-length and processed Relish, we favor the hypothesis that Relish degradation is regulated by the $SCF^{SkpA/dCul1/Slimb}$ complex. Further studies of Relish ubiquitination patterns and degradation profiles in wild-type and *SkpA* mutant flies are required to directly test our hypothesis.

Previous genetic studies provided several insights into the mechanisms that negatively regulate the Toll pathway: mutations in *cactus* induce both embryonic ventralization and the expression of antimicrobial peptide genes in the fat body, demonstrating Cactus function as an inhibitor of Dorsal and DIF. Similarly, mutations in *necrotic*, which encodes a serine protease inhibitor, lead to constitutive cleavage of the Toll ligand, Spaetzle, and activation of the Toll pathway, indicating that a serine protease pathway mediates Spaetzle cleavage in response to infection [34]. Our analysis of antimicrobial gene expression in SCF complex and proteasome mutants provides the first information on repression of the IMD pathway. This negative regulation appears to be mediated through turnover of both full-length Relish and the processed Rel-homology domain that localizes to the nucleus [9, 10]. Consequently, this may also be a mechanism for maintaining the expression patterns of *Drosophila* immune genes that show acute expression profiles after infection [35, 36]. *Diptericin* expression in bacterially infected $SkpA^{G49}$ mutants, for example, is not attenuated as rapidly as in infected wild-type flies (Figure 2A). Although SCF complex-mediated degradation of transcription factors is an established pathway for regulating gene expression [37], there is no previous evidence for an SCF complex regulating NF-κB in the nucleus. One mechanism proposed for NF-κB downregulation in mammalian cells is the acetylation of nuclear-localized RelA/P65 followed by binding to IκB and export to the cytoplasm [38]. A recent report, however, suggests that RelB is degraded in mammalian nuclei [39], and our results provide evidence that a similar mechanism, mediated by an SCF complex, may downregulate NF-κB in *Drosophila*.

Conclusion

In a screen for negative regulators of the *Drosophila* IMD signaling pathway, we isolated two mutations in the *SkpA* gene that encode a component of the *Drosophila* $SCF^{SkpA/dCul1/Slimb}$ ubiquitin ligase complex. Genetic analysis demonstrates that the ubiquitin-proteasome system represses the IMD pathway. RNAi studies suggest that the target of the $SCF^{SkpA/dCul1/Slimb}$ complex is the NF-κB

homolog, Relish, that functions in the IMD pathway. Consequently, we hypothesize that proteolytic degradation may be one mechanism for downregulating NF-κB activity in *Drosophila*.

Experimental Procedures

Drosophila Strains

Oregon^R flies were used as a wild-type reference. The *imd*^{1} mutation is described in [3], *Drosomycin* expression in the *Toll*^{10b} mutation is described in [21], the *slimb*^{1} mutation is described in [27], and the *DTS5* mutation is described in [28]. The *UAS-DTS5* line is described in [30], and the *c564-Gal4* driver, which is expressed in larval fat body cells, is described in [7]. The *UAS-Relish* line is described in [2]. *dCullin1*$^{l(2)02074}$ is a P-element insertion in the *dCullin1* gene that is maintained in the Bloomington stock center collection. *Relish*^{E20}, *Dmlkk*γ^{key1} (*kenny*^{1}), *Dredd*^{B118}, *Dmlkk*β^{ird5} (*ird5*^{1}), and *dTak1*^{1} are either strong or null alleles of *Relish*, *DmIKK*γ, *Dredd*, *Dmlkk*β, and *dTak1*, respectively [2, 5, 6, 7, 12]. *Diptericin-GFP* is a P-element transgene containing a fusion between 2.2 kb of upstream sequence from the *Diptericin* gene and the coding sequences from the *Green Fluorescent Protein* (*GFP*) gene [20]. *Drosomycin-GFP* is a P-element transgene containing a fusion between the *GFP* gene and 2.4 kb of upstream sequence from the *Drosomycin* gene [40]. *Drosophila* stocks were maintained at 25°C. After infection, flies were incubated at 29°C.

EMS Mutagenesis and Screen

y,w; *diptericin-GFP* male flies were treated with 25 mM EMS. The mutagenized males were crossed to *C(1)DX y,w,f* females, and the resulting F1 males were screened for constitutive *Diptericin-GFP* expression. *GFP*-expressing males were backcrossed to the same female stock to generate independent lines. For each line, a homozygous stock was established by crossing F2 males with *FM3/l(1)44ter* females. Initial mapping was performed by screening recombinants between the mutated chromosome and a chromosome carrying the y^+, *cv*, and *f* markers. Additional mapping was done with the X chromosome-deficiency kit from the Bloomington stock center.

Infection Experiments

Bacterial and fungal infections were performed as previously described [35].

Cloning and Sequencing the *SkpA* Alleles

Genomic DNA isolation from all strains and plasmid rescue experiments from the two P-element insertion lines, *l(1)G0389* and *l(1)G0109*, were performed by using protocols obtained from the Berkeley *Drosophila* Genome Project website: http://www.fruitfly.org/about/methods/index.html. Specific oligonucleotides for the *SkpA* gene were synthesized and were used to amplify the *SkpA* coding sequence. The resulting single-fragment PCR products were purified with a Qiagen purification column and were sequenced with the Bigdye Terminator Cycle sequencing ready reaction (PE Applied Biosystems). The sequence profiles were analyzed on Edit View 1.0.1 ABI Prism (PE Applied Biosystems). *SkpA* sequences from the *y,w* strain and the *SkpA*^{J6} and *SkpA*^{G49} mutants were compared with the *SkpA* sequence deposited in GenBank as accession number AF220066: the *SkpA*^{J6} mutation replaces cytosine 3087 with a thymine nucleotide, and the *SkpA*^{G49} mutation replaces guanine 3095 with an adenine nucleotide.

Northern Blot Analysis

Total RNA extractions, RNA gel blots, and quantifications were performed as previously described [21].

S2 Cell Culture and Assay Conditions and RNAi

Culture and treatment of S2 cells and double-stranded RNAi preparation is as previously described [10]. Primers used to generate templates for the double-stranded RNAs are provided as Supplementary Material available with this article online.

Western Blot Analysis

Total protein extractions and protein gel blots were performed as previously described [10].

Supplementary Material

Supplementary Material including the primer sequences used to generate DNA templates for gene-specific double-stranded RNA preparation is available at http://images.cellpress.com/supmat/supmatin.htm.

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